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Characterization of Two Novel Neuropeptides From the Sea Cucumber *Holothuria glaberrima*

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Abstract. Two peptides were purified from intestinal extracts of a sea cucumber, *Holothuria glaberrima,* by high pressure liquid chromatography (HPLC). The peptides were detected by a radioimmunoassay (RIA) based on an antiserum raised to the molluscan peptide, pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (pQDPFLRFamide). Automated sequencing and mass spectrometry indicate that the isolated peptides are: Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2 (GFSKLYFamide) and Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2 (SGYSVLYFamide). These are the first peptides to have been isolated from a member of the echinoderm class Holothuroidea.

The antiserum used in the RIA of the peptides was also employed in localizing immunoreactive nerve cells and fibers in the intestine of *H. glaberrima.* The immunohistochemical results suggest that these peptides might be responsible for the FMRFamide-like immunoreactivity reported earlier. Sequence similarities between GFSKLYFamide, SGYSVLYFamide, and a pair of peptides previously isolated from starfish lead us to propose that all four molecules are members of a family of peptides acting as neurotransmitters in echinoderms.

Introduction

Very few echinoderm neuropeptides have been characterized. For example, the sequence of the first neuropeptide detected in this phylum—*i.e.,* gonad-stimulating substance (GSS) from starfish (Chaet and McConnaughy, 1959)—is still unknown (references in Cobb, 1988). Recently, FMRFamide-like immunoreactivity was detected in the nervous system of the starfish *Asterias rubens,* and immunoreactive nerve fibers were found in the area of the tube feet, suggesting that FMRFamide might be regulating the process of locomotion (Elphick *et al.,* 1989). Subsequently, two novel neuropeptides from the starfish *A. rubens* and *A. forbesi* were identified: GFNSALM-Famide and SGPYSFNSGLTFamide, and the previously reported FMRFamide-like immunoreactivity in *A. rubens* was attributed to these peptides (Elphick *et al.,* 1991).

Peptide immunoreactivity has also been demonstrated in members of another echinoderm class, the Holothuroidea. For example, cholecystokinin (CCK)-like immunoreactivity occurs in neurons and in a plexus of fibers in the intestines of *Holothuria mexicana, Holothuria glaberrima,* and *Stichopus badionotus* (García-Arrarás *et al.,* 1991a). Similarly, FMRFamide-like immunoreactivity was reported in cells and fibers of the intestine of *H. glaberrima* (García-Arrarás *et al.,* 1991b). The location of these immunoreactive substances suggests that they have a role in the regulation of digestive physiology, and indeed peptides of the CCK family do induce a partial relaxation of the intestinal musculature (García-Arrarás *et al.,* 1991a). These results notwithstanding, none of the endogenous peptides in the nervous system of sea cucumbers had been identified before the present study was undertaken.

In this report, we describe the isolation and characterization of two peptides from the digestive system of *H. glaberrima.* In addition, we provide histochemical evidence for the presence of these peptides in the enteric nervous system of the sea cucumber.

Materials and Methods

Specimens of *H. glaberrima* (10–15 cm in length) were collected from the rocky intertidal zone of the north coast

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of Puerto Rico. The animals were either used immediately or, in some cases, maintained in marine aquaria at the Department of Biology of the University of Puerto Rico.

Digestive system extracts

Four extracts of sea cucumber digestive systems were prepared as follows. First, 13 to 25 animals were sectioned with a razor blade, just posterior to the calcareous ring. The body wall was slit open, and the intestinal tract, including the esophagus and the small and large intestine, together with adherent pieces of hemal vessel and respiratory tree, were removed. The tissue (37–113 g wet weight) was placed in a fourfold excess of acetone and left at −20°C for 48 h. The supernatant was then filtered through Whatman #1 paper, and the acetone and part of the water were removed on a rotary evaporator. The aqueous portion was acidified to 0.02 *M* acetic acid, centrifuged at 2500 × *g*, and the supernatant dried in a Speed-Vac (Savant). The dried sample was reconstituted in aqueous 0.1% trifluoroacetic acid (TFA), centrifuged, and filtered.

Purification

The reconstituted sample was pumped onto a Brownlee C8 reverse phase column (Aquapore RP300: 220 × 4.6 mm or Prep 10 Aquapore Octyl 100 × 10 mm), according to the procedure described by Price *et al.* (1990a). Once loaded, the column was rinsed with aqueous solvent (0.1% TFA) until the UV absorbance fell to near baseline. The eluting solvent was rapidly changed (by a step or 1 min gradient) to 20 or 30% of organic solvent (80% acetonitrile containing 0.1% TFA), whereupon a linear gradient was started with a 1%/min increase in the organic solvent up to 50 or 60% organic. Half-minute fractions were collected, and 2 μl aliquots were taken from each fraction for the RIA.

Further purification was also done on Brownlee C8 RP-300 columns (220 × 2.1 mm or 220 × 4.6 mm). The columns were developed, either with TFA/acetonitrile gradients as above, or with aqueous 0.05% heptafluorobutyric acid (HFBA) and 80% acetonitrile containing 0.05% HFBA.

Radioimmunoassay (RIA)

A rabbit antiserum (Q2), raised against pQDP-FLRFamide coupled to thyroglobulin (Price *et al.*, 1990b), was diluted 1:500 for use in the RIA. Iodinated pQYPFLRFamide served as the tracer.

Sequencing and spectrometry

The most immunoreactive fraction within each pure peak was analyzed. The fraction was divided in half: one half was dried in a Speed-Vac for FABms, and the other half (about .1 ml) was applied (in 3 portions with intermediate drying) directly to a pre-conditioned glass-fiber filter disk containing 3 mg of Polybrene. The disk was placed in the sequencer (Applied Biosystems 470A gas-phase sequencer with an on-line 120a PTH analyzer), and the PTH-amino acid derivatives in each cycle were identified by their retention times and quantitated by comparison of the peak areas to standards (performed by B. Parten, University of Florida Interdisciplinary Center for Biotechnology Research, Protein Sequencing Core Facility, Gainesville). The FABms analysis was carried out on a JEOL HX100HF magnetic sector mass spectrometer, as described in Bulloch *et al.* (1988).

Synthetic peptide

The peptides GFSKLYFamide and SGYSVLYFamide were synthesized on an Applied Biosystems synthesizer by the Protein Chemistry Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research; t-Boc protecting groups were used. The peptides were deprotected and removed from the resin with trifluoromethanesulfonic acid (Applied Biosystems protocol), purified by HPLC, and quantified by amino acid analysis (Hitachi 835 analyzer).

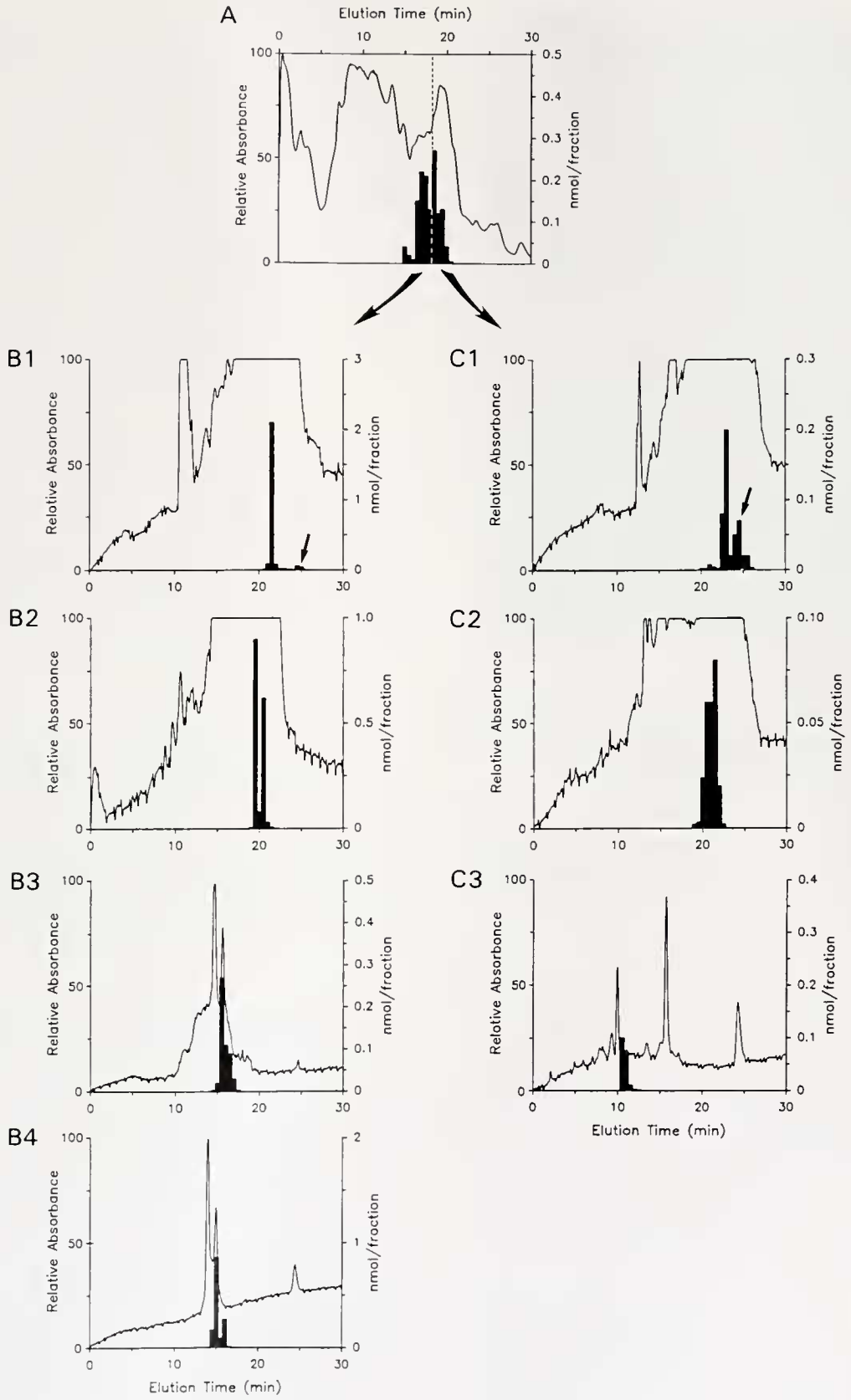
Immunohistochemistry

For the histochemical study, the procedure described by García-Arrarás *et al.* (1991a) was followed. In brief, isolated portions of the large and small intestines of *H. glaberrima* were fixed in picric acid-formaldehyde mixture overnight at 4°C. The tissue sections (10–12 μm) were treated with antiserum Q2 (1:500) or with an antiserum against FMRFamide (#8 3i 2s; 1:500) (García-Arrarás *et al.*, 1991b). As a control, the Q2 antiserum was incubated with 10 μg/ml of GFSKLYFamide, FMRFamide (Peninsula), or FLRFamide (Sigma) for 24 h before being applied to the tissue sections.

Results

Fractionation of extracts

Each of the four gut extracts was fractionated with a somewhat different series of HPLC steps. We discovered, finally, that the simplest way to purify the immunoreactive peptides was to select the immunoreactive fractions after each step, and to run them back through the same column under the same conditions (Fig. 1). This finding was certainly not expected. The method works because the extract behaves anomalously; *i.e.*, the immunoreactive peaks shift to an ever earlier elution time during each successive step of purification (*e.g.*, compare Figs. 1C2 and 1C3). Moreover, even the order of elution of the immunoreactive


A
Elution Time (min)
Relative Absorbance
nmol/fraction
B1
B2
B3
B4
C1
C2
C3
Elution Time (min)

peaks changes (Fig. 1). This relative shift in elution position was clearly observed in all but the first of the four extracts.

One peptide, already purified from the first extract, was readily and unambiguously identified as GFSKLYFamide when we obtained its molecular ion (860.4) and sequence (Fig. 2a). The calculated value of the amide of this sequence is 860.5, whereas that of the free acid is 861.5. Thus the molecular ion confirmed the presence of the C-terminal amide, which had been inferred from the immunoreactivity; in contrast, the PTH derivatives of phenylalanine and its amide are indistinguishable in normal Edman sequencing. The first extract contained other minor immunoreactive peaks; one of these was analyzed by FABms and sequenced; this product, found again in the fourth extract, will be described further below.

From the second extract, we obtained a molecular ion (934.6) and the partial sequence of a second peptide, SGXSVLXFamide (where X could be either tyrosine or methionine sulfone). The first peptide (GFSKLYFamide) did not appear in this extract.

The third extract contained two immunoreactive peaks. Using mass spectrometry, we again identified GFSKLYFamide (860 molecular ion) and SGXSVLXFamide (934 molecular ion).

The fractionation of the fourth extract was undertaken to resolve the ambiguities left after the first three, and the HPLC runs leading to the purification of the two most immunoreactive peaks are shown in Figure 1.

The first HPLC step in this last purification yielded a broad, irregular peak of immunoreactivity (Fig. 1A). From the earlier half of this, we succeeded, after four steps, in purifying a peak (at 15 min in Fig. 1B4) that sequenced as SGYSVLYF (Fig. 2B). The calculated molecular ion for this peptide—with its C-terminal amidated—is 934.5, and this is in good agreement with the molecular ion (934.6) found earlier. The small immunoreactive peak at 16 min in Figure 1B4 had the same sequence as the main peak, so it is probably just a tail of the main peak.

From the later half of the initial broad immunoreactive peak obtained in the first step of the purification (Fig. 1A), two peaks were resolved in the second (Fig. 1C1). The major (and earlier-eluting) of these peaks co-eluted with synthetic GFSKLYFamide.

The second peak, eluting at 23.5–25 min, was pooled with a similar small peak that had eluted a few minutes after SGYSVLYFamide (see arrows in Figs. 1B1 and 1C1). After purification (not shown), these pooled minor peaks yielded the sequence GFSXLYF, which corresponds to that of the synthetic peptide, except that no lysine (or other PTH derivative) appeared in cycle 4. This peak had a molecular ion of 958, which is 98 larger than that expected for the lysine containing amidated peptide. A peak with the same relative elution time, and a similar molec-

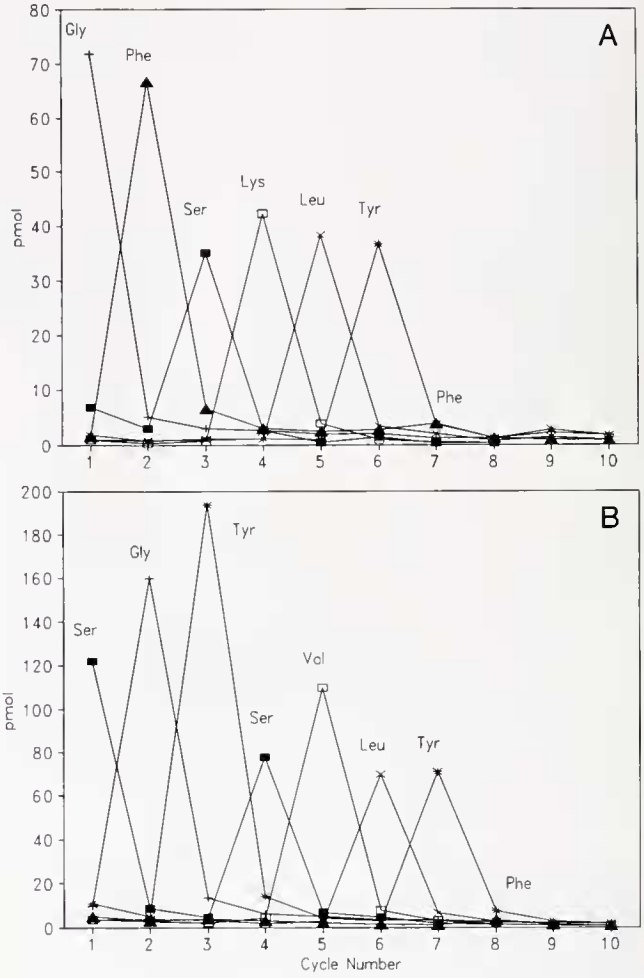


Figure 2. Amino acid sequences of the purified peptides. The yields of the pertinent PTH amino acid derivatives at each cycle are plotted and the amino acid assigned to each position is shown. (A) The peptide having an 860 molecular ion. (B) The peptide having a 934 molecular ion.

Figure 1. HPLC fractionation of a sea cucumber gut extract. The ultraviolet absorbance at 210 nm (solid line) and the immunoreactivity (histogram) are shown for each HPLC run. The initial fractionation (A) was done on a Prep10 Octyl column (10 × 100 mm; 4 ml/min) with a 30 min gradient from 16 to 40% acetonitrile in water with 0.1% trifluoroacetic acid. Subsequent runs (B1-4; C1-3) were done on an RP-300 column (2.1 × 220 mm; 0.5 ml/min) with the same gradient. The arrows in B1 and C1 indicate peaks that were pooled and subsequently purified (not shown; see text). The full scale absorbance in the top trace is 2.0 and is 0.5 AU in all the subsequent traces.

ular ion (958.6) and sequence, had also appeared in the first extract, as briefly noted above.

We suspect that the peptide GFSXLYFamide has a derivatized lysine side chain. For example, a peptide with the lysine amino group amidated by hexanoic acid would give such a molecular ion, and such a derivative could occur naturally in the sea cucumber. It is more likely, however, that the derivative is a substituted oxazolidine ring, formed by the condensation, with the lysine, of two molecules of acetone (58 + 58) with the loss of a water (−18). This derivatization would add 98 to the molecular weight of the peptide. In summary, this peptide may very well be an artifact of the purification.

Synthetic peptides

The synthetic peptides GFSKLYFamide and SGYSVLYFamide were purified by HPLC after deprotection. Each peptide co-eluted with its natural counterpart on HPLC. An amino acid analysis of GFSKLYFamide gave the following composition: Gly 1.05, Phe 1.75, Ser 1.0, Lys 1.1, Leu 1.25, and Tyr 1.05. The composition of SGYSVLYFamide was: Gly 1.00, Phe .90, Ser 2.03, Leu 1.11, Tyr 1.99, Val 1.33.

Q2 immunoreactivity

The tissue distribution of immunoreactivity to Q2 (the antiserum used in the characterization of GFSKLYFamide and SGYSVLYFamide) was determined by immunohistochemistry. Results with antibodies Q2 and #8 (the latter an antiserum against FMRFamide) were similar. Cells and fibers located in the outer connective tissue (serosa) of the small and large intestine were labeled, as were single fiber-like projections in the submucosal layer (Fig. 3). In addition, a strong nerve plexus was observed in the mesentery next to the muscular layer. This nerve plexus was continuous with the serosal nerve plexus. In contrast to the FMRFamide-like immunoreactivity (García-Arrarás *et al.*, 1991b), Q2 also labeled a group of cells located in the submucosal layer of the intestines, similar to what has been described as morula cells (Hetzel, 1963, 1965), but this reaction does not seem to be specific.

When the Q2 antiserum was preabsorbed with GFSKLYFamide (12 μM), no immunoreactivity to Q2 was observed in the cells and fibers of the mesentery, serosa, or submucosa; but the morula cells continued to be labeled. These results suggest that other antibodies in the Q2 serum recognize unrelated substances. The peptides FMRFamide (17 μM) and FLRFamide (17 μM) were also used for preabsorption of Q2, but they could not completely block the observed immunoreactivity of the cells and nerve fibers in the serosa, or of the nerve fibers in the submucosa. The Q2 immunoreactivity of the morula cells was not blocked, whether the antiserum was preabsorbed with FMRFamide or with FLRFamide.

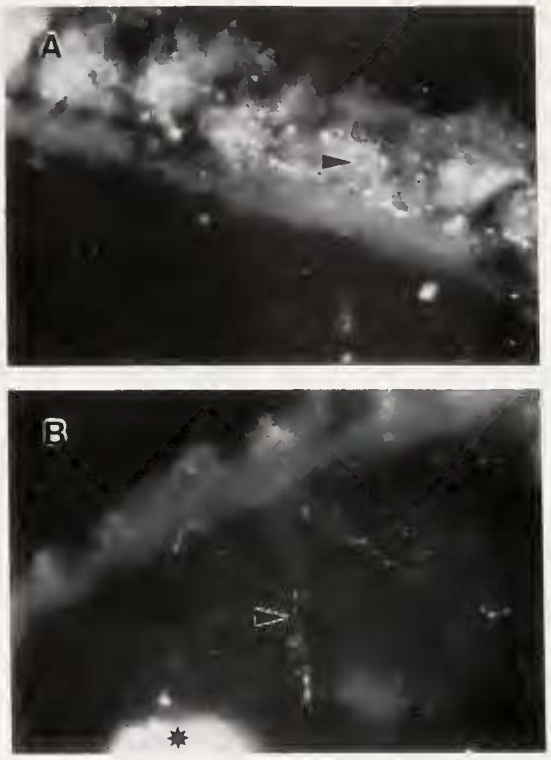


Figure 3. Transverse sections of the large intestine of *H. glaberrima* showing Q2-like immunoreactivity. A. Immunoreactive nerve fibers at the level of the serosa and longitudinal muscle. Most of these fibers were associated with the longitudinal muscle (arrowhead). B. Arrowhead points at one immunoreactive fiber extending throughout the submucosa layer. Asterisk: endogenous fluorescence. Magnification: A. ×405; B. ×270.

Discussion

We have purified two peptides from gut extracts of the sea cucumber *H. glaberrima,* using high pressure liquid chromatography for separation and radioimmunoassay for detection. These peptides—GFSKLYFamide and SGYSVLYFamide—were completely characterized by microsequencing and mass spectrometry, and are the first to have been isolated from the echinoderm class Holothuroidea.

Two related peptides were isolated earlier from another echinoderm class, the Asteroidea: *i.e.,* GFNSALMFamide and SGPYSFNSGLTFamide from the starfishes *Asterias forbesi* and *A. rubens* (Table I; Elphick *et al.*, 1991). These peptides, like those of the sea cucumber reported here, were detected on the basis of their binding to an antiserum, Q2, raised against pQDPFLRFamide (Price *et al.*, 1990b). In both studies, furthermore, the Q2 antiserum was originally selected because the aim was to characterize putative FMRFamide-related peptides (FaRPs) that had been

Table I

Amino acid sequences of SxLxFamide[1] peptides isolated from Echinodermata

CLASS Species	Sequence	Ref.
HOLOTHUROIDEA		
Holothuria glaberrima	Gly-Phe-**SER**-Lys-**LEU**-Tyr-**PHE**-NH_2	2
	Ser-Gly-Tyr-**SER**-Val-**LEU**-Tyr-**PHE**-NH_2	
ASTEROIDEA		
Asterias forbesi	Gly-Phe-Asn-**SER**-Ala-**LEU**-Met-**PHE**-NH_2	3
A. rubens	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-**SER**-Gly-**LEU**-Thr-**PHE**-NH_2	

[1] Ser-x-Leu-x-Phe-NH_2, where the positions "x" can be occupied by any residue.
[2] This report.
[3] Elphick *et al.*, 1991.

identified by immunocytochemistry (Elphick *et al.*, 1989; García-Arrarás *et al.*, 1991b).

FaRPs, defined liberally, have now been isolated from many animal groups, including coelenterates, nematodes, annelids, arthropods, and even vertebrates (reviewed by Price and Greenberg, 1989; Greenberg and Price, 1992), and a penultimate arginyl residue is not only a common feature of this extended family, but has been shown to be critical for physiological activity (*e.g.*, see Kobayashi and Muneoka, 1986). Antiserum Q2 should have identified most FaRPs that might have been present in our extracts, but in fact, not one of the four neuropeptides sequenced from echinoderms has the penultimate arginine. Our results, therefore, when taken together with the evidence obtained from the starfish (Elphick *et al.*, 1991), suggest that authentic FaRPs are absent from the Echinodermata.

If the above assertion is correct, we must account for the FMRFamide-like immunoreactivity described in sea cucumbers (García-Arrarás *et al.*, 1991b) and in starfish (Elphick *et al.*, 1989). In the sea cucumber *H. glaberrima*, immunoreactivity to FMRFamide has been reported in the radial nerves, in nerve plexuses of the esophagus, and in the enteric nervous system (García-Arrarás *et al.*, 1991b). The distribution of immunoreactivity observed with the Q2 antibody in the gut of *H. glaberrima* is similar, if not identical, to that observed with antibodies against FMRFamide. The localization of Q2-binding to nerve cells and fibers in the sea cucumber intestine suggests that the peptides recognized by this antibody occur in the enteric nervous system of holothurians, and that they may be involved in neural transmission or modulation.

The story in the starfish *Asterias rubens* is similar: *i.e.*, FMRFamide-like immunoreactivity was detected in the radial nerve cords, the circumoral nerve ring, and the subepithelial nerve plexus of the tube feet (Elphick *et al.*, 1989), and this immunoreactivity has been attributed to the two isolated peptides from the starfishes by Elphick *et al.* (1991). In conclusion, the FMRFamide-like immunoreactivity previously reported in both echinoderm classes might be due to the presence of the isolated peptides reacting with the anti-FMRFamide serum.

As Table I illustrates, the two sea cucumber peptides have five of seven residues in common, and the two starfish peptides have five of eight identical; but the most similar sea cucumber and starfish peptides share only four of eight residues. The clear unifying feature of these four echinoderm peptides is the C-terminal sequence SxLx-Famide, where the positions indicated by "x" can be occupied by any other residue. We therefore propose that this periodic sequence of serine, leucine, and phenylalanine defines a novel family of peptides present in the Echinodermata.

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